Persistent Clonal Proliferation of Human T-lymphotropic Virus Type I-infected Cells in Vivo

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ABSTRACT

Clonal proliferation of human T-lymphotropic virus type 1 (HTLV-I)-infected cells has been detected by Southern blot analysis and inverse PCR in patients with adult T-cell leukemia, patients with HTLV-I-associated diseases, and even in asymptomatic carriers. Combining inverse PCR with long PCR, we amplified the genomic DNA regions flanking the integration sites of the HTLV-I provirus to detect clones of infected cells. Inverse long PCR revealed that increased virus load was associated with an increase of both the number of cells in each clone and the number of clones. Clonal proliferations were found in both CD4- and CD8-positive cells in a carrier and a patient with HTLV-I-associated neuropathy/tropical spastic paraparesis. These HTLV-I-infected clones persisted over several years in the same carriers, and, moreover, most of the persistent clones were CD4 positive in a HTLV-I carrier. These findings indicate that HTLV-I infection plays an important role in the clonal expansion of lymphocytes and the prolonged survival of CD4-positive cells in vivo. Surviving T-lymphocytes may be susceptible to genetic changes, leading to the onset of leukemia.

INTRODUCTION

After ATL, was identified as a distinct clinical entity (1, 2), HTLV-I was identified as the causative agent of ATL (3, 4). It was the first retrovirus shown to be associated with a human disease. Thereafter, HTLV-I was revealed to be associated with not only ATL but also with various other diseases, such as HAM/TSP (5, 6), HU (7), infective dermatitis (8), and arthropathy (9). Thus, HTLV-I causes these inflammatory diseases, as well as neoplastic disease, but the mechanism by which it causes these diseases remains unknown.

The virus load was reported to differ more than 100-fold in HTLV-I carriers (10). The intermediate state was reported as a clinical condition associated with an increased number of HTLV-I-infected cells, often complicated by opportunistic infection, such as Strongyloides infection or mycosis, and the intermediate state was also considered as a preleukemic state (11) because two patients with intermediate state had ATL during follow-up. An increase in number of HTLV-I-infected cells was also reported in patients with HAM/TSP (12) and HU (13) compared with asymptomatic carriers. HTLV-I-infected lymphocytes were also found in the spinal cord of patients with HAM/TSP and in the eye of patients with HU, indicating a close relationship.

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Received 3/24/97; accepted 9/5/97.

The abbreviations used are: ATL, adult T-cell leukemia; HTLV-I, human T-lymphotropic virus type I; HAM, HTLV-I-associated neuropathy; TSP, tropical spastic paraparesis; HU, HTLV-I uveitis; IL PCR, inverse long PCR; PBMC, peripheral blood mononuclear cell.

DIFFERENCES AMONG HTLV-I-Infected CLONES

Inverse PCR revealed that HTLV-I provirus was quantitated by the method described by Ono et al. (18). HTLV-I provirus was quantitated by the method described by Ono et al. (18).

IL PCR. To amplify the genomic DNA adjacent to the integration sites of the HTLV-I provirus, we used IL PCR (Fig. 1). First, genomic DNA (500 ng) was digested with EcoRI, which does not cut HTLV-I provirus. The DNA was ligated by T4-DNA ligase and then digested with MluI, which digests the X region of HTLV-I. This step prevented amplification of the HTLV-I provirus itself. The resulting DNA was used as a substrate for long PCR. We performed long PCR as described (19) using the XL PCR kit (Perkin-Elmer, Branchburg, NJ) and a Robocycler (Stratagene, La Jolla, CA) according to manufacturer's protocol. Briefly, primers (final concentration, 10 μM), MgCl2 (1.1 mM), dNTPs (0.2 mM) and XL buffer were mixed in a total volume of 20 μl, and AmpliWax (Perkin-Elmer Corp.) was added. The wax was melted by incubating the reaction tube at 80°C for 5 min. After this step, tubes were cooled to room temperature, and then substrate DNA (20 μl), XL buffer (9 μl), and rTth DNA polymerase XL (1 μl) were added in a volume of 30 μl. We performed 33 or 37 cycles of amplification as follows: denaturation at 94°C for 30 s and annealing and extension at 64°C for 5 min. Primers used in this experiment were as follows: primer 1 in the U5 region of LTR (positions 566–589), 5'-GGTTCAGACCTGTGTTCTCAACTCTACGTC3'; primer 2 in the U3 region (positions 8345–8378), 5'-AGTCTGGCCCTGGACCTTTTCAGACTCTGTTTCC3'. PCR products (5 μl) were electrophoresed in a 1% agarose gel containing ethidium bromide.

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Southern Hybridization and Sequencing. PCR products were electrophoresed in a 1% agarose gel and transferred to a nylon membrane. This filter was hybridized with radiolabeled oligonucleotides derived from the pX region and the LTR as follows: pX probe (positions 7336–7355), 5'-CGGATACCCAGTCTACGTGT-3'; LTR probe (positions 728–747), 5'-CCAGCGACAGCCCATCCTCAT-3'. We subcloned PCR products into pCR II using TA cloning kits (Invitrogen Corporation, San Diego, CA) and sequenced the plasmid DNAs by the dideoxy method using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, OH) according to the manufacturer’s protocol.

RESULTS

IL PCR. IL PCR was performed as described in “Patients and Methods” (Fig. 1). Because a previous report described the stochasticity of detection of clonality by inverse PCR (20), we studied HTLV-I carriers with a high virus load (about 32,500 copies/75,000 PBMCs; 50%) and a low virus load (about 150 copies/75,000 PBMCs: 0.2%) with various numbers of amplification cycles (33 and 37 cycles; Fig. 2). In a carrier with a high virus load, most bands were repeatedly detected in all three assays (Fig. 2, Lanes 1–6), but some faint bands differed among experiments. The same results were obtained in other carriers with a high virus load. On the other hand, we could not detect any bands with 33-cycle PCR in carriers with a low virus load (Fig. 2, Lanes 7–9), but with 37-cycle PCR, we identified different bands in each experiment (Lanes 10–12). These results showed that IL PCR was random and nonreproducible for minor clones, but there were some dense bands that were consistently detected among experiments, which indicated major clones. Dilution experiments with plasmid DNA showed that 37-cycle PCR detected only a few copies of HTLV-I, but at least 50 DNA copies were needed for detection by 33-cycle PCR (data not shown). Therefore, we performed IL PCR analyses with only 33 cycles to ensure that the detected bands represented the clonal proliferation of HTLV-I-infected cells and to prevent the detection of minor clones.

Southern blot analysis confirmed that an internal probe (U5 region oligonucleotide probe) hybridized to all PCR-generated bands in eight carriers, whereas a pX probe could hybridize to only one or two bands, confirming that most of these bands were derived from
Table 1. Sequences flanking the integration sites of HTLV-I provirus amplified by IL PCR

<table>
<thead>
<tr>
<th>5'-genomic region</th>
<th>5'-LTR</th>
<th>3'-LTR</th>
<th>3'-genomic region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAAGCATTAGTAGATGGACACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>TTAAGAAGGATTATGTGACAATGACCATGA</td>
<td>AAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>GAGCCACCATACCTGTGACAATGACCATGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>CTAAAAGTAATAAACTGACAATGACCATGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>AGGCCCATTAGTTTTTTTAGACAACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>TTTAAGATAGATATCAGACCATAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>GATCTCCTTTCTCTCGATGAACTATGGCACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>GAATTAGTAGTAGATGGACACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>GATGGGAATATGGACACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>CTGACAATATTTGACACTAGA</td>
<td>GAAAATGATACACATAGTATAAGATCAGT</td>
<td>-</td>
</tr>
</tbody>
</table>

Flanking sequences of 5'- and 3'-LTR of IL PCR products are shown, and repeated sequences, which are characteristic of integration sites, are underlined. Viral sequences are shown in boldface type.

Fig. 2. IL PCR analysis of carriers with high and low virus loads. Genomic DNAs derived from carriers with high (50%) and low (0.2%) virus loads were used for IL PCR with 33 and 37 cycles in triplicate.

As shown in Fig. 2, most of the PCR products were less than 4 kb long, indicating that large DNA fragments were difficult to amplify in this assay. Furthermore, this method could not amplify the integration sites when the genomic DNA flanking HTLV-I contained MluI sites or, in the case of a defective virus, had only one LTR (19). Using IL PCR, we studied 17 patients with ATL and found major clones, which were thought to be leukemic clones, in 8 of 17. This indicated that IL PCR could detect about one-half of HTLV-I-infected clones because of the limitations stated above. Nevertheless, this assay can detect many clones in HTLV-I carriers and is more reproducible (Fig. 2) when compared with previous studies using inverse PCR (15, 17). The use of high temperature for annealing and extension of long PCR was thought to enable this reproducibility. Our previous study with inverse PCR showed clonal proliferation of infected cells in patients with ATL, but several different bands were amplified in different experiments using samples from some HTLV-I carriers. This indicated the presence of several different clones of HTLV-I-infected cells in HTLV-I carriers, and nested PCR picked up different clones in each experiment. Unlike the other methods (16, 17), IL PCR is convenient because there is no need for a radioisotope or any special apparatus.

Virus Load and Clonal Proliferation. To analyze the relationship between virus load and clonality, we studied carriers with various virus loads using IL PCR (Fig. 3). Only one major band was detected in an ATL patient. In carriers with a high virus load, many clones were present, along with a few denser bands. Only a few bands were visible in carriers with a low virus load. In some carriers, the IL PCR method could not detect the integration site of the HTLV-I provirus,

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We analyzed the clonal proliferation of HTLV-I-infected cells in 58 HTLV-I carriers and 33 patients with HTLV-I-associated diseases using the IL PCR method. Clonal proliferation of HTLV-I-infected cells was detected in not only CD4-positive cells but also CD8-positive cells in a carrier and a patient with HAM/TSP, and infected clones in the carriers survived for several years.

Clonal proliferation of HTLV-I-infected cells in HTLV-I carriers was reported by our group and other investigators (15–17). Wattel et al. (16) described clonal proliferation of HTLV-I-infected cells in eight individuals (patients with HAM/TSP or HTLV-I carriers) using linker-mediated PCR. It was speculated that clonal proliferation increased virus load because of remarkable stability of env sequence in two carriers (16). Because pX sequences were more variable in carriers than env sequences (22), whether virus load is increased by only clonal proliferation or an increase in the number of clones of infected T-cells contributes to increased virus load remains unclear. The increase that we found in the number of clones detected may reflect more major (i.e., proliferating) clones detectable by IL PCR. This study is the first to show that many clones persist over several years, an observation that supports the hypothesis that increased virus load is mainly caused by clonal proliferation.

Furukawa et al. (23) reported the frequent detection of clonal proliferation of HTLV-I-infected cells in patients with HAM/TSP and their family members by the Southern blot method. Because increased virus load was reported in patients with HAM/TSP, it is likely that only a few major clones were detected among many clones, most of which were undetected by Southern blot analysis. The fact that HTLV-I-infected clones could be detected by Southern blotting confirms that some major clones existed in these patients.

Detection of long-surviving HTLV-I-infected cells by IL PCR suggests that viral proteins, especially Tax, may immortalize infected lymphocytes (24, 25). These viral proteins have been revealed to interact with cellular genes associated with signal transduction or proliferation of T-lymphocytes, including interleukin 2 and its receptors. Tax has also been shown to have an inhibitory effect on apoptosis.

**Fig. 3.** Correlation between virus load and clonal proliferation. The clonal proliferation of HTLV-I-infected cells in an ATL patient and in HTLV-I carriers with various virus loads (shown as percentages) was analyzed by IL PCR (33 cycles). Numbers represent the percentages of HTLV-I-infected cells if one cell is assumed to have one copy of the HTLV-I provirus. HL-60, a HTLV-I-negative human myeloid cell line, was used as negative control.

indicating that infected cells were very rare (less than 0.1% of cells were infected). These findings suggest that virus load is determined by both the number of cells in each clone and the number of clones. We analyzed 53 asymptomatic carriers, 17 patients with ATL, 13 with HAM/TSP, and 3 with HU. Clonal proliferation was also detected in patients with HAM/TSP or HU, and their patterns were not different from asymptomatic carriers (data not shown).

**Clonal Proliferation of HTLV-I-infected Cells Continued for Years.** We detected clonal proliferation of HTLV-I-infected cells in HTLV-I carriers; the next question is how long such clones survive in vivo. To answer this question, we studied sequential DNA samples (2–7 years) of nine carriers in a Miyazaki cohort study. Representative results from three carriers are shown in Fig. 4. Most HTLV-I-infected clones survived for several years in the HTLV-I carriers shown, and this result was also obtained from the other carriers. It should be noted that dense bands, representing major clones, were detected repeatedly during the time course. As discussed previously, changes in faint bands may be due to the stochasticity of the method.

HTLV-I can infect not only CD4-positive cells but also CD8-positive cells (21). The subset in which clonal proliferation is detected remains unknown. CD4- and CD8-positive cells were sorted from a carrier with a high virus load (case 4) and a patient with HAM/TSP (case 5) using immunoaffinity beads, and then the genomic DNAs were analyzed by IL PCR (Fig. 5). Different bands were found in CD4- and CD8-positive cells in these cases, showing that clonal proliferation occurred in both subsets. Sequential analysis in case 4 showed that most of the persistent clones were CD4 positive (Fig. 5, solid arrows), whereas CD8-positive clones fluctuated during the time course (open arrowheads) in a carrier. This observation showed that HTLV-I is associated with clonal proliferation of both CD4- and CD8-positive cells, but the persistence of clones may differ between the subsets.

**Fig. 4.** Sequential analysis by IL PCR. Genomic DNAs from three different carriers at different time points were analyzed by IL PCR (33 cycles). The year when the samples were collected is shown at the top of each lane.
case 4) and a patient with HAM/TSP (case 5) were analyzed by IL PCR. The month and year when the PBMCs were collected is shown at the top of each lane. Solid arrows, clonal proliferation of CD4-positive cells; open arrowheads, clones of CD8-positive cells.

The immortalization of T-lymphocytes, especially CD4-positive T-lymphocytes, is thought to be important in the pathogenesis of HTLV-I-associated diseases and the leukemogenesis of ATL. It also suggests that long-surviving T-lymphocytes accumulate somatic changes of oncogenes or tumor-suppressor genes (30, 31), leading to the onset of leukemia. This hypothesis would also explain the long latency (usually several decades) of ATL after infection.

HTLV-I was reported to infect CD4-positive lymphocytes, CD8-positive cells, B cells, and monocytes (21), but clonal proliferation in each subset was not analyzed in previous studies. Although the clonal proliferation of monocytes and B cells remains to be studied, this study shows that clonal proliferation occurs in both CD4- and CD8-positive cells, and persistent clones were predominantly CD4 positive, at least in one HTLV-I carrier. This finding is consistent with the observation that HTLV-I preferentially immortalizes CD4-positive lymphocytes in vitro. It is possible that viral proteins, such as Tax, may contribute to long survival of CD4-positive cells by interaction with cellular proteins.

The virus load differs more than 100-fold among carriers, but does not change in each individual during follow-up over several years, indicating that long-surviving clones may be the molecular basis of stable virus load. What mechanism controls this clonal proliferation and determines the virus load in HTLV-I carriers? The immune response, including cellular immunity and humoral immunity, may be responsible for controlling the number of HTLV-I-infected cells. Sonoda et al. (32) reported that different HLA haplotypes were associated with HAM/TSP and ATL. It is possible that carriers with high CTL activity against cells presenting viral peptides suppress the number of HTLV-I-infected cells, and carriers with low CTL activity allow the proliferation of infected cells.

In this study, we analyzed the clonal expansion of HTLV-I-infected cells using IL PCR and showed that clonal cells persisted over several years. This is the first report to demonstrate the long survival of HTLV-I-infected T-lymphocytes in vivo. Such surviving CD4-positive T-lymphocytes are implicated in the pathogenesis of inflammatory diseases and presumably in the leukemogenesis of ATL. Further studies to elucidate the mechanism to cause clonal expansion of HTLV-I-infected cells may shed light on the pathogenesis of HTLV-I-associated inflammatory diseases and ATL, and also on the prevention and therapy of these diseases.

ACKNOWLEDGMENTS

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REFERENCES

7. Rocha, M., Watanabe, T., Yamaguchi, K., Takatsuki, K., Yoshimura, K., Shinra,

(26) Suzuki et al. (27) reported that Tax binds to p16, which is a cell cycle inhibitor, resulting in immortalization of cells. Expression of the tax gene in HTLV-I carriers was detected by reverse transcriptase-mediated PCR, and anti-Tax antibodies were detected in carriers (28, 29). These findings showed that the tax gene was expressed in vivo at least in a small number of cells, and may be associated with the expansion of CD4-positive cells by the mechanism discussed above. The immortalization of T-lymphocytes, especially CD4-positive T-lymphocytes, is thought to be important in the pathogenesis of HTLV-I-associated diseases and the leukemogenesis of ATL. It also suggests that long-surviving T-lymphocytes accumulate somatic changes of oncogenes or tumor-suppressor genes (30, 31), leading to the onset of leukemia. This hypothesis would also explain the long latency (usually several decades) of ATL after infection.

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